

Preliminary evidence of reductive stress in human cytotoxic T-cells following exercise

Wadley, Alex; Holliday, Adrian; Morgan, Rhys G.; Heesom, Kate J.; Aldred, Sarah; Peters, Derek M.; Bueno, Allain ; Coles, Steven

DOI:

[10.1152/jappphysiol.01137.2017](https://doi.org/10.1152/jappphysiol.01137.2017)

License:

Other (please specify with Rights Statement)

Document Version

Peer reviewed version

Citation for published version (Harvard):

Wadley, A, Holliday, A, Morgan, RG, Heesom, KJ, Aldred, S, Peters, DM, Bueno, A & Coles, S 2018, 'Preliminary evidence of reductive stress in human cytotoxic T-cells following exercise', *Journal of Applied Physiology*. <https://doi.org/10.1152/jappphysiol.01137.2017>

[Link to publication on Research at Birmingham portal](#)

Publisher Rights Statement:

Published in *Journal of Applied Physiology* on 12/04/2018

DOI: 10.1152/jappphysiol.01137.2017

General rights

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

- Users may freely distribute the URL that is used to identify this publication.
- Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
- User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
- Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

**Preliminary evidence of *reductive stress* in human cytotoxic T-cells
following exercise**

Alex J. Wadley^{1,2*}, *Adrian Holliday*³, *Rhys G. Morgan*⁴, *Kate J. Heesom*⁵, *Sarah Aldred*⁶,
Derek M. Peters^{7,8}, *Allain A. Bueno*¹ & *Steven J. Coles*¹

¹ Institute of Science & the Environment, University of Worcester, Worcestershire, WR2 6AJ

² School Sport, Exercise & Health Sciences, Loughborough University, Loughborough, LE11
3TU, UK

³ Institute of Sport, Physical Activity and Leisure, Leeds Beckett University, LS1 3HE

⁴ School of Cellular and Molecular Medicine, University of Bristol, Bristol, BS8 1TD

⁵ Proteomics Facility, University of Bristol, Bristol, BS8 1TD.

⁶ School of Sport, Exercise and Rehabilitation Sciences, University of Birmingham, B15 2TT

⁷ Institute of Health & Society, University of Worcester, Worcestershire, WR2 6AJ

⁸ Faculty of Health & Sport Sciences, University of Agder, Kristiansand, Norway

* Current Institution

Keywords: Redox, immunity, lymphocyte, physical activity, reactive oxygen species

Running Title: Reductive stress in immune cells after acute exercise

Word Count: 4710

Corresponding author

Dr Steven J. Coles

Institute of Science and the Environment

University of Worcester, St Johns Campus, Henwick Grove

Worcester, UK, WR2 6AJ

Email: s.coles@worc.ac.uk

Phone: 01905 542577

Abstract

This study investigated immunophenotypic differences in intracellular thiol redox state of peripheral blood mononuclear cells (PBMCs) isolated from trained (TR, n=9, mean \pm SD: age 28 ± 5 years; BMI 23.2 ± 2.6 kg·m²; $\dot{V}O_{2max}$ 56.9 ± 6.1 ml·kg⁻¹·min⁻¹) and recreationally active (RA, n=11, mean \pm SD: age 27 ± 6 years; BMI 24.2 ± 3.7 kg·m²; $\dot{V}O_{2max}$ 45.1 ± 6.4 ml·kg⁻¹·min⁻¹) participants before and after a maximal aerobic exercise tolerance test. Blood samples were taken before (PRE), during (sample acquired at 70% HR_{max}), immediately (POST+0) and 15 minutes post-exercise (POST+15). PBMCs were isolated and reduced thiol analysis (fluorescein-5 maleimide (F5M)) by immunophenotype (CD3⁺, CD4⁺ and CD8⁺) was performed using flow cytometry. A significant increase in cellular F5M fluorescence was observed in CD3⁺ T-cells at POST+0, with changes driven to a greater extent by CD8⁺ T-cells (fold change in both groups CD4: +2.3, CD8: +2.8; p<0.05). Further analysis revealed a population of highly reduced CD8⁺ T-cells (*CD8⁺T-reduced⁺*) that significantly increased from PRE to POST+0 in RA participants only (RA: +272 cell/ μ L, p<0.05). To further understand these results, *CD8⁺T-reduced⁺* and *CD8⁺T-reduced⁻* cells were analysed for immunophenotype in response to the same exercise protocol (n=6, mean \pm SD: age 24 ± 5 years; BMI 25.7 ± 4.1 kg·m²; $\dot{V}O_{2max}$ 41.33 ± 7.63 ml·kg⁻¹·min⁻¹). *CD8⁺T-reduced⁺* had significantly less lymphoid homing potential (CCR7) POST+0 compared to PRE. This study is the first to demonstrate that lymphocyte populations become more *reductive* in response to acute exercise.

New & Noteworthy

The study presented provides the first evidence to suggest that cytotoxic T-cells become transiently reductive in healthy individuals following a single bout of cycling. Detection of these cells was enabled via the use of a flow cytometric assay that incorporates the thiol reactive probe Fluorescein-5 maleimide. Using this method, transient *reductive stress* in viable T-cells is permissible and provides the basis for further research in the area of exercise immunology.

Abbreviations

ANOVA: Analysis of Variance, BMI: Body mass index, CV: Coefficient of variance, CD: Cluster of differentiation, CCR7: Chemokine receptor type 7, DTNB: 5,5'-dithiobis (2-nitrobenzoic) acid, EDTA: ethylene diaminetetraacetic acid, FCS: Fetal Calf Serum, F5M: Fluorescein-5 maleimide, HR_{max}: Maximum heart rate, GSH: Glutathione, GSSG: Glutathione disulphide, GTP: Guanidine triphosphate, HBSS: Hanks Balance Salt Solution, H₂O₂: Hydrogen peroxide, IL: Interleukin, IPAQ: International Physical Activity Questionnaire, NAC: N-acetylcysteine, NADPH: Nicotinamide adenine dinucleotide phosphate, NEM: N-ethylmaleimide, PD-1: Programmed death receptor-1, PBMC: Peripheral Blood Mononuclear Cell, PBS: Phosphate buffered saline, PRDX: Peroxiredoxin, Pre: Blood sample taken before exercise, Post+0: Blood sample taken immediately post-exercise, Post+15: Blood sample taken 15 minutes post-exercise, RONS: Reactive oxygen and nitrogen species, RPM: repetitions per minute, RPMI: Roswell Park Memorial Institute, SD: Standard deviation, -SH: Sulphur-hydryl, TNB: 5-thio-2-nitrobenzoic acid, TR: Trained, VPA: Vigorous physical activity, VCO₂: Carbon dioxide consumption, $\dot{V}O_2$: Oxygen uptake, $\dot{V}O_{2\text{ MAX}}$: Maximum oxygen uptake.

Introduction

Alterations in peripheral blood immune cell subpopulations during exercise are well characterised, with transient increases in effector CD8⁺ T-cells and Natural Killer cells observed at the expense of CD4⁺ T-cells and B lymphocytes (4). However, the precise mechanisms driving mobilization and tissue extravasation of these cell types are unclear. Studies have demonstrated that global protein oxidation increases after acute exercise, with redox enzyme activity and protein content (i.e. superoxide dismutase, glutathione peroxidase and peroxiredoxin) concurrently increased (8, 43–45, 48). This suggests that redox balance may have a role in the regulation of the immune system after exercise.

Exercise is known to induce the production of reactive oxygen and nitrogen species (RONS), a diverse group of ubiquitous reactive molecules that are widely implicated in mediating cell and tissue function during exercise (11, 19, 23, 29, 30). *In vitro* evidence suggests that cell permeable RONS such as hydrogen peroxide (H₂O₂) can reversibly oxidise nucleophilic cysteine thiol groups that are conserved within various cellular proteins (51). These include antioxidant enzymes such as thioredoxin and peroxiredoxin (39, 52) as well as various signaling proteins, such as GTPases, phosphatases and transcription factors (33). The oxidation (i.e. sulfenylation/ sulfinylation) of thiol-containing proteins has been previously demonstrated in total lymphocyte pools following acute exercise (44, 48). This could be a potential mechanism by which H₂O₂ triggers redox-sensitive signaling pathways and/ or elicits oxidative stress, which may in turn influence immune cell mobilization and immunophenotype. Interestingly, recent studies have demonstrated that supplementation with the thiol donor, N-acetylcysteine (NAC) can blunt immune cell mobilization (i.e. macrophages and lymphocytes) patterns in response to both muscle-damaging (24, 31) and exhaustive aerobic exercise (28). This drives the hypothesis that shifts in thiol redox state may regulate immunoregulation.

Previous studies which have evaluated redox changes in protein thiols following acute exercise have relied on isolating all immune cells from peripheral blood (44, 48), through which downstream analysis is performed using lysates from a heterogeneous cell populations. These techniques, whilst informative, do not permit evaluation of cellular thiol redox state within individual cells of a specific immunophenotype. Furthermore, these analytical procedures require cell lysis for their proteomic approaches, which could lead to artificial thiol oxidation, making data interpretation a challenge. Previous studies have utilized the thiol specific probe, fluorescein-5 maleimide (F5M) to label and identify intracellular proteins with solvent accessible reduced cysteines in cell line models using immunoblotting techniques (35, 53). In this respect, a loss of fluorescent signal relative to background controls is indicative of thiol oxidation i.e. an increased cellular *oxidative stress*, whereas a gain in signal would indicate *reductive stress*. Interestingly, F5M is excited at $\lambda 488$ nm and emits at $\lambda 525$ nm (38), offering the potential for F5M to be incorporated into flow cytometric immunophenotyping assays. Thus by adapting previous protocols that have used F5M to monitor intracellular thiol redox changes (7, 35, 53), flow cytometry would represent a powerful option to monitor *oxidative* and *reductive* stress by immunophenotype in a variety of contexts, e.g. following exercise. For example, cells of the adaptive immune system i.e. T-helper ($CD4^+$) and T-cytotoxic ($CD8^+$) lymphocytes are important to evaluate in this context, since oxidative stress has been documented in these cells post-exercise (8, 43–45, 48). Both types of T-lymphocyte are antigen specific, where $CD4^+$ T-cells serve broadly to orchestrate the immune response via cytokine production and $CD8^+$ T-cells kill infected cells through the release of cytotoxic granules (50). Whilst the importance of *oxidative stress* in $CD4^+$ and $CD8^+$ T-cells following exercise is appreciated, the role of *reductive stress* in this context is currently unknown. The impact of chronic *reductive* stress is now being appreciated in the literature (1, 20, 22), however the potential immunological significance of this response is

unclear, specifically in relation to transient changes which may take place in response to exercise.

The aim of the present study was to monitor thiol-mediated redox changes in viable CD4⁺ and CD8⁺ T-cells isolated from individuals before and after a cycling ramp test to exhaustion. Following optimization of a flow cytometric protocol, thiol mediated redox changes in these lymphocytes were quantified, and further studies carried out to characterise these cells with regards to immunophenotype and lymphoid homing potential.

Materials and methods

Participants

Following ethical approval from the University of Worcester Research Ethics Committee, twenty healthy males were initially recruited to take part in the study. Trained individuals (TR, n=9, mean \pm SD: age 28 ± 5 years; BMI 23.2 ± 2.6 kg.m²; $\dot{V}O_{2max}$ 56.9 ± 6.1 ml.kg⁻¹.min⁻¹) were recruited from local athletics, cycling and triathlon clubs. The criterion for "trained" was regularly completing in a minimum of 3 hours of endurance training per week, for a minimum of 2 years and a maximal oxygen consumption of at least 50 ml.kg⁻¹.min⁻¹(2). Recreationally active individuals (RA, n=11, mean \pm SD: age 27 ± 6 years; BMI 24.2 ± 3.7 kg.m²; $\dot{V}O_{2max}$ 45.1 ± 6.4 ml.kg⁻¹.min⁻¹) were recruited through adverts at the University of Worcester. All participants completed questionnaires addressing health history, and habitual levels of weekly physical activity (mean \pm SD: TR 10235 ± 6394 ; RA 4932 ± 2555) and vigorous physical activity (VPA, mean \pm SD: TR 800 ± 1008 ; RA 264 ± 271) were assessed by the International Physical Activity Questionnaire (IPAQ). Based on the results, a further six recreationally active participants (n=6, mean \pm SD: age 24 ± 5 years; BMI 25.7 ± 4.1 kg.m²; $\dot{V}O_{2max}$ 41.33 ± 7.63 ml.kg⁻¹.min⁻¹) were recruited to characterise the CD8⁺T-

149 *reduced*⁺ cell population.

150 All participants gave their written informed consent and the study was carried out in
151 accordance with the Declaration of Helsinki (2008). Participants were non-smokers and
152 reported that they had not taken any antioxidant vitamin supplements or anti-inflammatory
153 drugs for 4 weeks prior to the laboratory visit. In addition, participants reported to be free
154 from any viral or bacterial infections for at least 4 weeks prior to taking part. Participants
155 were also required to refrain from any strenuous physical activity, consumption of alcoholic
156 beverages or caffeine for two days prior to the experimental session.

157 158 Experimental session

159 All experimental sessions took place at the University of Worcester in the morning
160 (7.00-7.30am start time), following at least a 10 hour fast. After a thirty-minute period of rest,
161 resting heart rate and blood pressure (*Omron Healthcare, Hoofddorp, The Netherlands*),
162 height (*Seca Alpha, Hamburg, Germany*) and mass (*Tanita, Tokyo, Japan*) were determined.
163 Cardiorespiratory fitness ($\dot{V}O_{2\max}$) was then measured using a ramp exercise test to
164 exhaustion on an electromagnetically braked cycle ergometer (*Lode Excalibur Sport,*
165 *Groningen, Netherlands*). Workload commenced at 50 Watts and was increased by 1 Watt
166 every 6 seconds, until volitional exhaustion or until cadence fell below 60 revolutions per
167 minute. Oxygen uptake was assessed continuously using a breath-by-breath system (*Oxycon*
168 *Prx, Jaeger, Wuerzburg, Germany*) and heart rate monitored using a Polar Vantage heart rate
169 monitor (*Polar Vantage, Kempele, Finland*). The breath-by-breath system was calibrated
170 daily or before each test to verify gas levels using a fixed volume cylinder (3L), a gas canister
171 of known composition (4.5% CO₂, 3.5% O₂ and 5.0% N₂), as well as adjusting for any subtle
172 atmospheric changes. A final obtained value of rate of oxygen consumption relative to body
173 mass was accepted as $\dot{V}O_{2\max}$ (ml.kg⁻¹min⁻¹) if two of the following criteria were met in

conjunction with a plateau in oxygen consumption with an increase in work load: volitional exhaustion; a respiratory exchange ratio of ≥ 1.15 ; heart rate within $10 \text{ beats} \cdot \text{min}^{-1}$ of the age-predicted maximal heart rate ($220 - \text{age}$) (16). Upon completion of the exercise test, participants rested in a seated position for 15 minutes before the final blood sample was obtained.

Blood sampling

A catheter (*Appleton Woods, Birmingham, UK*) was inserted into the antecubital vein of the arm prior to exercise to obtain a baseline sample after thirty minutes of rest (Pre). A second blood sample was taken when participants reached predicted 70% HR_{max} during the exercise test. Subsequent blood samples were taken immediately (Post+0) and 15 minutes post-exercise (Post+15). At each time point, 12 ml of blood was drawn into two separate vacutainer tubes containing potassium ethylene diaminetetraacetic acid (EDTA) (*Becton, Dickson & Company, Oxford, UK*). The catheter was flushed every 30 minutes with isotonic saline solution (0.9% sodium chloride) to prevent blood clotting. For the characterisation of $\text{CD8}^+ \text{T-reduced}^+$ (n=6), two blood samples were obtained (12ml) via venepuncture to the antecubital vein of the arm prior to (Pre) and immediately following exercise (Post+0) only.

Flow cytometry method validation

Jurkat immortalised T-cell line (*ATCC, Middlesex, UK*) was maintained in RPMI-1640 adjusted to contain 10% (v/v) foetal calf serum, 2 mM L-glutamine and 100 U/ml penicillin /100 mg/ml streptomycin. Redox balance was perturbed by adding 0.1 mU/mL glucose oxidase to 2×10^6 Jurkat cells (14). The cells were incubated for four hours with samples taken at the start (T0), after 1 hour (T1) and after 4 hours (T4). The samples were washed with phosphate buffered saline (PBS) and then incubated for 20 minutes with F5M

(0.1 μ M) in the dark at 4°C, followed by two washes with FACS buffer. The concentration of F5M was determined through titrations (0-10 μ M) during pilot analysis to optimise the fluorescent signal on FL-1 FITC (data not shown). The F5M-labelled cells were subjected to flow cytometric analysis (*Guava easyCyte, Millipore UK Ltd, Hertfordshire, UK*). F5M MFI was monitored by the λ 488 nm laser and λ 525/30nm detector (FL1 channel). Cell viability was evaluated using trypan blue.

Blood cell isolation and sample preparation

Whole blood from each time point was used to isolate peripheral blood mononuclear cells (PBMCs) using density gradient centrifugation. Blood was diluted 1:1 with Hanks Balance Salt Solution (HBSS), and then layered carefully on top of Ficoll paque PLUS (*GE Healthcare, Buckinghamshire, UK*), before centrifuging at 400g for 40 minutes at 21°C. The PBMC layer was aspirated and then washed three times with HBSS, by centrifuging steps at 300g for 10 minutes.

Approximately one million cells per time point were used for flow cytometry analysis of intracellular reduced thiol content using F5M. As part of the immunophenotypic characterisation analysis, 4×10^6 /ml cells from each time point were incubated with 50 μ M N-ethylmaleimide (NEM, oxidised glutathione, GSSG), and an additional 4×10^6 /ml cells with PBS (total glutathione) for 20 minutes at 4°C to determine reduced: oxidised glutathione ratios. Cells were washed twice with FACS buffer (PBS supplemented with 2% (v/v) foetal calf serum, 0.02% sodium azide (v/v) and 5 mM EDTA) and then stored at -80 °C.

Flow cytometry and confocal microscopy analysis

Approximately 200,000 viable PBMCs were used for identification of reduced cellular thiols in specific lymphocyte populations using four-colour flow cytometry (*Guava*

easyCyte, Millipore UK Ltd, Hertfordshire, UK). Cells were incubated with fluorescently conjugated antibodies CD3-PE (clone: HIT3b), CD3-APC (clone: HIT3a) CD4-APC (clone: RPA-T4), CD8-APC (clone: HIT8a), CD8-PE (clone: SK1), CD279-PerCP (clone: EH12.2H7), CD197- PerCP (clone: G043H7) and CD28-APC (clone: CD28.2) (*Biolegend*, Cambridge, UK) for 30 minutes at 4°C followed by intermittent washes with PBS for 5 minutes at 300 x g. Cells were then incubated for 20 minutes with F5M in the dark (0.1 µM) at 4°C, followed by two washes with FACS buffer. NEM (20 µM) was used as an experimental control to confirm thiol-binding specificity. Confocal microscopy analysis was performed to confirm that F5M had acquiesced within viable T-cells. Cells treated with F5M (0.1 µM) were stained with CD3-APC (as above). Fluorescent cells were visualised using the resonant scanning head of a TCS SP2 confocal laser microscope (*Leica*, Buckinghamshire, UK), under a x 63 oil immersion objective NA 1.32 (HCX-PL-APO). Isotype-matched control and NEM treated cells were used to determine the fluorescence threshold for the helium-neon (CD3-APC) and argon (F5M) laser respectively.

Glutathione ratio assay

Reduced: oxidised ratios of GSH:GSSG in PBMCs were quantified using a commercially available kit (*Sigma Aldrich*, CS0260, Dorset, UK), following minor modifications. Samples were prepared as described above (n=6). On the day of analysis, NEM (GSSG) and PBS-stained pellets (total glutathione) were thawed on ice and then 50 µL of Sulfosalicylic acid (5%) added to each pellet. Cells were rapidly freeze-thawed in liquid nitrogen and a 37°C water bath twice respectively, followed by centrifugation at 10,000 g for 10 minutes. The supernatant was extracted and then loaded (10 µL) onto a 96-well microtiter plate with known reduced glutathione (GSH) standards (0-50 µM) in duplicate. A working solution (150 µL) of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, 1.5 mg/ml) and glutathione

reductase (6 units/ ml) was added to each well and left for 5 minutes at room temperature. Nicotinamide adenine dinucleotide phosphate (NADPH, 50 μ L) was then added to each well, and the rate of DTNB reduction to 2-nitro-5-thiobenzoate (TNB) monitored by recording the change in absorbance every minute for 5 minutes at λ 412. Nanomoles of GSH in each sample were determined for both NEM and PBS pre-incubated samples to determine GSSG and total glutathione concentrations respectively. GSH concentration was determined by subtracting GSSG from total glutathione concentration, and a subsequent ratio of GSH: GSSG used to determine half-cell reduction potential (Ehc/ mV) using the Nernst equation (36).

Data analysis

Flow cytometry data were analysed using GuavaSoft 3.1 (*Millipore UK Ltd, Hertfordshire, UK*). Background fluorescence was established by isotope-matched controls. Lymphocytes were identified based on forward versus side scatter and T-cells determined as being either CD3⁺ CD4⁺ (T-helper) or CD3⁺ CD8⁺ (T-cytotoxic). The CD4:CD8 ratio was determined by determining the percentage of cells within the CD4⁺ and CD8⁺ regions. Within each T-cell region, a distinct population of cells with high F5M fluorescence were determined (*CD4⁺T-Reduced⁺* and *CD8⁺T-Reduced⁺*). Changes in these populations were adjusted for total lymphocyte count and events within the CD3⁺, CD4⁺ and CD8⁺ gates and thus expressed as cells/ μ L. For each lymphocyte population, F5M mean fluorescence intensity (MFI) was normalised relative to pre-exercise (i.e. pre = 1), indicative of basal redox state. For the immunophenotypic characterisation analysis, lymphocytes were gated on forward versus side scatter and CD3⁺ CD8⁺ cells identified. *CD8⁺T-Reduced⁺* cells were distinguished from *CD8⁺T-Reduced⁻* cells, based on F5M binding and MFI changes in programmed cell death protein-1 (PD-1), CD28 and chemokine receptor-7 (CCR7) determined.

Statistical analysis

The Kolmogorov Smirnov test was used to test for normality in scale data at all time points and variables were accordingly log transformed if necessary. Differences between participant characteristics and the physiological responses to exercise were assessed using unpaired samples T-tests. The influence of training status (Trained and Recreationally active) on CD4:CD8 ratio and F5M signal were assessed over time (Pre, During, Post+0 and Post+15) by a 2*4 repeated-measures analysis of variance (ANOVA) for each of the different T-cell pools (Lymphocytes, CD3⁺, CD3⁺CD4⁺, CD3⁺CD8⁺, CD3⁺CD4⁺T-Reduced⁺, CD3⁺CD4⁺T-Reduced⁻, CD3⁺CD8⁺T-Reduced⁺ and CD3⁺CD8⁺T-Reduced⁻). Post hoc analysis of any interaction effects (Group*Time) was performed by a test of simple effects by pairwise comparisons, with Bonferroni correction. GSH:GSSG ratios and MFI changes in CCR7 and CD28 over time (Pre and Post) were assessed by paired samples T-tests. MFI changes for PD-1 over time were assessed by the Wilcoxon Signed Rank Test.

All values are presented as means \pm standard deviation or error (indicated throughout manuscript). Data in figures are not reported as log transformed values. Statistical significance was accepted at the $p < 0.05$ level. Statistical analyses were performed using SPSS (*PASW Statistics, release 23.0, SPSS Inc., Chicago, IL, USA*).

Results

Flow cytometric assay optimization

Jurkat cells exposed to 0.1 mU/ml glucose oxidase showed a F5M MFI increase from 1059 ± 203 (n=3) to 1569 ± 380 (n=3) after 1 hour (T1) compared with the start (T0). After 4 hours of incubation (T4) the F5M MFI decreased to 788 ± 211 (n=3) (Figure 1A). Normalised values (T_n/T_0) illustrate an increase in F5M at T1 compared to T0 and a decrease at T4

(Figure 1B). Since F5M reacts specifically with reduced thiols, these data indicate *reductive* stress at T1 and oxidative stress at T4 (Figure 1B). Treatment with glucose oxidase had no overall impact on cell viability (data not shown).

Physiological responses to the exercise test

To assess the physiological responses of TR and RA groups to exercise, $\dot{V}O_{2\max}$, power output, heart rate and test duration were measured and reported in Table 1. All participants achieved $\dot{V}O_{2\max}$ with regards to our criteria. TR had a significantly greater $\dot{V}O_{2\max}$ ($p=0.001$) and maximal power output relative to body mass ($p=0.003$) than RA. No significant differences in test duration, timing of the blood sample during exercise or peak heart rate were noted between TR and RA. There were no statistically significant differences in the physiological responses between the two RA cohorts when characterising lymphocyte thiols ($n=20$) and subsequent immunophenotyping ($n=6$).

Analysis of the lymphocyte composition of peripheral blood in response to exercise

Changes in the ratios of $CD4^+$ to $CD8^+$ T-cells are reported in Figure 2. A significant reduction in CD4:CD8 ratio was observed during and immediately post-exercise only in RA (Figure 2, Group*Time effect: $F_{3,54} = 3.77$, $p=0.016$). The ratio decreased at all time points relative to Pre ($p<0.0001$), and was lower at Post+0 relative to During ($p<0.0001$) in RA. In TR, the ratio was significantly lower only at Post+0 relative to Pre ($p=0.004$). The ratio increased Post+15, relative to Post+0 (TR $p=0.045$; RA $p=0.041$) in all participants, but was only significantly lower than Pre in RA ($p<0.0001$). At rest, CD4:CD8 ratio was higher in RA, relative to TR ($p=0.016$).

Analysis of intracellular reduced thiol content in lymphocytes by immunophenotype

Following this, we applied our flow cytometry method to determined intracellular reduced cysteine thiol fluorescence in viable human T-populations (Figure 3) in response to exercise (Figures 4 and 5). Using a hierarchical gating strategy, it was first determined that exercise caused an increase in the normalised F5M ratio at Post+0 in gated lymphocyte (time effect: $F_{3,54} = 4.2$, $p=0.010$) and $CD3^+$ pools (time effect: $F_{3,54} = 4.1$, $p=0.011$) in all participants. These changes were driven by $CD8^+$ (time effect: $F_{3,54} = 3.5$, $p=0.029$ – Figure 5A) and not $CD4^+$ T-cells (time effect: $F_{3,54} = 2.0$, $p=0.132$ – Figure 4A). There were no differences in F5M normalised ratios between RA and TR groups in either T-cell population.

Alterations in the $CD4^+T\text{-Reduced}^+$ and $CD8^+T\text{-Reduced}^+$ concentration are reported in Figures 4C and 5C. A significant increase in the concentration of $CD8^+T\text{-Reduced}^+$ cells at Post+0, relative to Pre ($p=0.003$) and During ($p=0.009$) were noted in recreationally active participants only (time*group interaction effect: $F_{3,54} = 3.821$, $p=0.015$). At Post+15, the concentration of $CD8^+T\text{-Reduced}^+$ cells was not greater than Pre ($p=0.216$). No differences were noted in the concentration of $CD4^+T\text{-Reduced}^+$ cells in either group.

Characterization of $CD8^+T\text{-Reduced}^+$

To further understand these observations, immunophenotypic characterization of the $CD8^+T\text{-Reduced}^+$ cells was carried out in a different sample of 6 individuals. Markers of T-cell activation (CD28), suppression (PD-1) and lymphoid homing (CCR7) were stratified according to the F5M signal before and after exercise. MFI changes in CD28, PD-1 and CCR7 are reported in Figure 6, with the exercise test data for this sample reported in Table 2. A significant decrease in CCR7 MFI was observed immediately after exercise in $CD8^+T\text{-}$

Reduced⁻ ($CD8^{+}T\text{-}Reduced^{+}$: $p=0.173$; $CD8^{+}T\text{-}Reduced^{-}$: $p=0.027$). However, $CD8^{+}T\text{-}Reduced^{+}$ exhibited a significantly lower CCR7 MFI relative to $CD8^{+}T\text{-}Reduced^{-}$ after exercise ($p=0.042$). PD-1 expression in gated $CD8^{+}$ cells was unchanged in response to exercise ($CD8^{+}T\text{-}Reduced^{+}$: $p=0.655$; $CD8^{+}T\text{-}Reduced^{-}$: $p=0.273$). PD-1 expression was lower in $CD8^{+}T\text{-}Reduced^{+}$, compared to $CD8^{+}T\text{-}Reduced^{-}$ at both time points but did not reach statistical significance (Pre-Exercise: $p=0.068$; Post-Exercise: $p=0.109$). No differences were noted in CD28 expression between the two populations.

Glutathione analysis

To identify a redox couple driving the observed *reductive* shift in cellular redox state, changes in the most abundant cellular redox couple, GSH:GSSG, was first quantified. The concentrations of GSH and GSSG were used to calculate an Ehc/ mV for the pool of isolated PBMCs using Nernst equations (36). No difference in PBMC Ehc/ mV was observed in response to exercise (pre: -209 ± 20 to post: -206 ± 16 . Ehc/ mV).

Discussion

The present study represents the first to investigate changes in lymphocyte solvent accessible reduced thiols in response to exhaustive exercise in healthy young males. Using the thiol-specific and fluorescent probe, F5M, we identified that the lymphocyte pool becomes transiently *reductive* in response to exercise. Within the lymphocyte pool, $CD8^{+}$ T-cells demonstrated a greater F5M fluorescence than $CD4^{+}$ T-cells immediately after exercise (Figures 4 and 5). At this time point, a population of $CD8^{+}$ T-cells was identified with high F5M binding capacity ($CD8^{+}T\text{-}reduced^{+}$), and it was demonstrated that the concentration of these cells was greater in recreationally active than aerobically trained participants. We

carried out further analysis to characterise immunophenotype specific exercise-induced changes in $CD8^+T\text{-reduced}^+$ in a separate group of recreationally active individuals. Collectively, the present data suggests that immediately after a single bout of exercise, peripheral blood is composed of more $CD8^+T\text{-reduced}^+$ cells that have a lower expression of CCR7, relative to $CD8^+T\text{-reduced}^-$.

It is well documented that exercise of sufficient intensity and duration can induce a transient increase in markers of *oxidative stress* (42, 43). As a result, multiple studies have reported elevated indices of oxidative stress following exercise in skeletal muscle (34, 46), blood cells (erythrocytes (3) and leukocytes(43, 48)) and extracellular fluids (plasma (49), saliva (6) and urine (37, 40)). The results of our study using an optimised flow cytometric approach provide evidence that the immune cell compartment of peripheral blood undergoes transient *reductive stress* after exercise. Of the 20 participants studied, 17 demonstrated a reductive shift in $CD8^+$ cell thiol redox state either immediately or 15 minutes after exercise (Figure 5). To our knowledge, reports of cellular *reductive stress* in humans have only been observed in chronic neurological (1, 20) and cardiovascular disease (25). Margaritelis et al (22) made the observation that many studies reporting mean trends in a specific extracellular marker of oxidative stress dismissed potential ‘reductive’ outliers as part of the dataset. In support of this, the present data supports the notion of acute intracellular *reductive stress* after exercise.

Determining the role of protein thiols as regulators of exercise modified redox balance is an evolving area of investigation (5). *Reductive stress* implies increased electron availability within the cell or a subcellular compartment whereby an abundant redox couple accepts more electrons. Although less abundant, signaling protein thiols may also become reduced in this context, altering key signaling pathways after exercise (47). Indeed, previous evidence has shown that the increased availability of low molecular weight *reduced* protein thiols such as

Glutathione (GSH) within T-cells facilitates their proliferation and secretion of Interleukin-2 (13). We quantified GSH:GSSG ratio to estimate changes in global thiol redox balance within PBMC lysates in response to exercise. Using Nernst equations based on Schafer et al (36), no changes were observed in PBMC mV after exercise (pre: -209 ± 20 to post: -206 ± 16 Ehc/mV). We performed further analysis as part of a proof of concept approach to label CD8⁺ T-cells with F5M and assess exercise-induced changes in solvent accessible cysteine thiol redox state, using mass spectrometry (see supplementary information). These preliminary data indicate that specific proteins can be identified (e.g. including transcription factors), and alterations in thiol redox state interpreted after exercise. Further analysis is required to identify the redox couples involved in the *reductive* shift in CD8⁺ T-cells. It must be emphasized that the results of the current study do not definitively pinpoint whether increased CD8⁺*T-reduced*⁺ cells are a result of cysteine thiol mobilisation or shifts in CD8⁺ cell immunophenotype. The former would imply higher intracellular reductive capacity that may result from an altered flux of glucose through the pentose phosphate pathway during exercise (41), thus increasing the cellular pool of NADPH, a cofactor for thiol reducing enzymes such as thioredoxin reductase (15). With regards to the immunophenotype, there is evidence to suggest that late differentiated CD8⁺ T-cells have higher basal thiol concentration than naive CD8⁺ T-cells (45). Late differentiated CD8⁺ T-cells have high effector function and are known to be highly responsive to exercise-induced mobilization (4). This would suggest that an increase in CD8⁺*T-reduced*⁺ cells could relate to an altered composition of CD8⁺ T-cells, rather than altered redox balance intracellularly. Only extensive immunophenotypic and functional analysis of CD8⁺ cells over a suitable time course would be able to determine the origin of the observed *reductive stress* in the immune cell compartment of peripheral blood.

The current data supports previous work showing an elevation in CD8⁺ T-cells in peripheral blood during and in the minutes following exercise (4), with the relative increase

in CD8⁺ cells exaggerated in individuals with lower training status (Figure 2). The CD4:CD8 ratio decreased During (70% HR_{max}) and Post+0 in RA, but only Post+0 in TR. This indicates that RA participants were more sensitive to CD8⁺ cell mobilization. In conjunction with this data, a novel finding herein was the identification of a population of CD8⁺ T-cells with a high capacity to bind F5M immediately post-exercise (CD8⁺*T-reduced*⁺, Figure 5B). The concentration of CD8⁺*T-reduced*⁺ cells present Post+0 increased from pre-exercise values in RA, with no change noted in TR individuals (Figure 5C). As a proportion of the CD8⁺ pool, CD8⁺*T-reduced*⁺ cells increased from 16 to 42% post-exercise in RA, with a 4% decrease noted in TR (data not shown). No differences were noted in CD4⁺*T-reduced*⁺ concentration or proportional composition between groups or in response to exercise (Figure 4C), suggesting that the *reductive* shift observed in CD8⁺ thiol redox state may relate to the mobilization patterns of immune cells in response to exercise.

To our knowledge, transient differences in intracellular redox state following exercise, based on training status have not been previously reported. However, there is evidence to suggest that physical training can upregulate the expression of endogenous antioxidant enzymes in both skeletal muscle (12, 18) and immune cells (42, 43). We can therefore hypothesize that enhanced antioxidant protection at rest may have therefore provided improved tolerance to exercise-induced ROS in the TR group and reduced the need for these individuals to mobilise CD8⁺*T-reduced*⁺ cells immediately after exercise. It must be noted that there were no statistical differences in total MFI changes in F5M between groups at Post+0, indicating that CD8⁺ cells in TR were also transiently *reductive*, however these individuals did not exhibit exercise-induced changes in CD8⁺*T-reduced*⁺ cell concentration. With regards to CD8⁺*T-reduced*⁺ cells, no correlation was noted between the concentration of CD8⁺*T-reduced*⁺ and CD8⁺ cells present in the circulation at Post+0 in RA (*data not shown*), suggesting no direct relationship between CD8⁺ mobilization and thiol redox state; however,

as mentioned above, this may be due to the precise immunophenotype of the CD8⁺ cells not being fully characterized. Nevertheless, our findings strengthen the growing body of evidence that suggests that *reduced* protein thiols may play an important role in the mobilization of specific subsets of immune cells after exercise, with training status of the individual being a factor worthy of further investigation.

Based on the above findings, an additional six recreationally active participants were recruited in order to characterize the immunophenotype of CD8⁺*T-reduced*⁺ cells in response to the same exercise protocol. We coupled F5M and antibody staining to compare alterations in thiol redox state with changes in cell markers of T-cell: activation (CD28), suppression (PD-1) and lymphoid homing (CCR7) before and immediately after exercise (Figure 6: B-D). CCR7 expression facilitates the migration of T-cells to secondary lymphoid organs i.e. lymph nodes, where interaction with other immune cells takes place. This process plays a central role in immune cell activation and clonal proliferation of antigen specific T-cells (9). A significant decrease in CCR7 expression was noted in CD8⁺*T-reduced* cells after exercise only (Figure 6C). Interestingly, CD8⁺*T-reduced*⁺ cells expressed lower levels of CCR7 than CD8⁺*T-reduced* cells post exercise. CCR7 is a lymphoid homing receptor expressed on naïve T-cells located in the primary lymphoid organs (i.e. thymus and bone marrow). Lower expression of CCR7 is known to mediate trafficking of effector T-cells towards secondary lymphoid organs (32). Therefore, given that CD8⁺*T-reduced*⁺ have less lymphoid homing capacity, but may be mobilised to a greater extent during exercise, these findings propose a role for redox-mediated driven immune cell mobilization and possibly, extravasation. No changes in CD28 expression were noted, suggesting that acute cellular *reduction* has little impact on co-stimulatory marker expression (Figure 6D). A trend for lower expression levels of the immunosuppressive cell-surface receptor, PD-1 was observed in CD8⁺*T-reduced*⁺ compared to CD8⁺*T-reduced* cells (p=0.068, Figure 6B). PD-1 is a cell surface receptor

expressed on activated T-cells that downregulates antigen-mediated T-cell activation. Interestingly, elevated PD-1 expression has been observed in many cancers (17, 21, 27), with subsequent T-cell exhaustion promoting poor anti-tumor responses.

We have validated a flow cytometry method *in vitro* (Figure 1), indicating that a standard physiological dose of enzyme-mediated H₂O₂ can elicit *oxidative* and *reductive* stress in Jurkat cells. This method has high utility for determining the behavior patterns of specific immune cell populations in response to exercise in humans. Further research is needed to expand on our findings regarding CCR7 and PD-1; to more accurately characterise the relationship between T-cell function, thiol redox state and the physiological relevance to exercise and immune function. Given that thiol labeling is achieved prior to cell lysis, advanced proteomic analysis can be used to determine modifications to solvent accessible thiol proteins that may alter cellular function (see supplementary information).

We must acknowledge some potential limitations to the present study. The application of F5M in highly diverse biological samples such as human PBMCS is subject to some subtle differences in membrane permeability, cross-reactivity with other nucleophiles (e.g. with primary and secondary amines) and probe hydrolysis, the latter two of which are pH dependent (pH>7.5). Decreases in lymphocyte pH (7.4) following a $\dot{V}O_{2\max}$ test would likely be minimal (10, 26), however, whereby our method is applied to more prolonged exercise models, subtle changes in pH should be accounted for. In line with this comment, it must also be noted that our results only reflect immune thiol redox state changes in response to a $\dot{V}O_{2\max}$ test, and not bouts of exercise that are more prolonged and sustained at moderate or high intensity. Future work is needed to clarify our current findings in response to more conventional types of exercise.

Conclusion

The present study provides evidence that the peripheral blood lymphocyte pool becomes transiently more *reductive* in response to acute exhaustive exercise in healthy males. Using the fluorescent probe, F5M we have identified a specific population of CD8⁺ T-cells (CD8⁺*T-reduced*⁺) that drives this reductive shift, with training status appearing to be a key variable differentiating individuals. We provide evidence to suggest that CD8⁺*T-reduced*⁺ may target secondary lymphoid organs post exercise and recommend that further research is undertaken to validate these observations, and characterise the significance of *oxidative* and *reductive* shifts in immune cell redox state post exercise.

Acknowledgements

AJW, SJC, AH and DMP were involved in the conception and design of the experiments. AJW, AH, KH and RGM carried out all data acquisition. Data analysis and interpretation was carried out by AJW, SJC, AH and DMP. Drafting of the article for important intellectual content was undertaken by AJW and all authors undertook revision and final approval of the manuscript. AJW, SJC, AH, SA, DMP and AAB provided biological insight. SJC provided project direction. We would like to acknowledge Jordan Vautrinot, Dr Tom Cullen, Owain Rowat, Ben Taisler, Alice Burgin, Matthew Davies and Matthew Holland at the University of Worcester for their assistance with data collection. We thank the University of Worcester for supporting this research.

Conflict of Interest

None of the authors declare a conflict of interest.

Funding

Tables

Table 1: Exercise physiology data (n=20): test duration, relative peak power output (Watt/kg), peak heart rate (bpm) and relative maximal oxygen consumption achieved during the exercise test. All values are means (standard deviation).

	Trained (n=9)	Recreationally Active (n=11)
Test Duration (min:sec)	27:31 (07:03)	22:44 (06:04)
Timing of During Exercise Sample (min:sec)	14:03 (03:35)	12:27 (04:06)
Relative Watt Max (Watt·kg ⁻¹)	4.24 (0.48) ^{\$\$}	3.45 (0.53)
Peak Heart Rate (beats·min ⁻¹)	185.89 (11.76)	189.36 (5.85)
Relative VO₂max (ml·kg ⁻¹ ·min ⁻¹)	56.93 (6.09) ^{\$}	45.10 (6.44)

Table 1 Legend: ^{\$} indicates a significant difference between trained and recreationally active groups (Group effect): ^{\$} p<0.05; ^{\$\$} p<0.001.

Table 2: Participant demographics and physiological data from participants taking part in the immunophenotyping analysis arm of the study (n=6): Values are means (standard deviation).

	n=6
Test Duration (min:sec)	18:07 (03:02)
Relative Watt Max (Watt·kg ⁻¹)	2.98 (0.60)
Peak Heart Rate (beats·min ⁻¹)	180.5 (9.67)

Relative VO_{2max} (ml·kg ⁻¹ ·min ⁻¹)	41.33 (7.63)
--	--------------

Fig 1: Optimisation of the F5M flow cytometric assay using Jurkat cells (n=3). (A)

Representative flow cytometric plot illustrating Jurkat cells labelled with F5M following glucose oxidase treatment. T0 = start, T1 = 1 hour incubation with glucose oxidase and T4 = 4 hours incubation with glucose oxidase. Intracellular thiol redox status was measured by F5M binding and a change in FL1-H (λ488nm; λ525/30nm) intensity. A cell sample unstained with F5M is also represented. (B) Summarised data illustrating normalised F5M signal (T_n/T₀). Indicated is ‘reduction’ i.e. more free cys-thiol relative to T0 and ‘oxidation’ i.e. less free cys-thiol relative to T0.

Fig 2: Changes in T-cell populations in response to acute exercise (n=20). Bars represent

CD4:CD8 ratio changes in RA (grey bars) and TR (white bars) Pre, During, Post+0 and Post+15. Values are means ± standard deviation. * indicates a significant difference relative to Pre: * p<0.05, *** p<0.0001. + indicates a significant difference relative to During: +++ p<0.0001. # indicates a significant difference relative to Post: # p<0.05. \$ indicates a significant difference between RA and TR (all Time*Group effects; pairwise comparisons).

Fig 3 Immunophenotypic assessment of reduced intracellular thiol content in viable T-

cell populations (n=20). (A) Representative gating strategy for CD8⁺ cells. Lymphocytes were gated (left panel) on forward light scatter (FSC) vs. side light scatter (SSC) and then stained for CD3+ (PE) and CD8⁺ (APC, right panel). (B) Intracellular reduced thiols were measured by a gain of F5M binding and increase in FL1-H (λ488nm; λ525/30nm) intensity. Unstained and an experimental control (NEM) were used to confirm thiol binding specificity

(C) A representative PBMC confocal image illustrating intracellular F5M signal (left image) and surface staining for T-cells with anti-human CD3-APC (right image).

Fig 4: Flow cytometric analysis of intracellular thiol redox status in CD4⁺ cells in response to acute exercise (n=20). (A) Bars represent normalised MFI on FL1-H (λ 488nm; λ 525/30nm) in gated CD4⁺ cells for RA (grey bars) and TR (white bars) participants. Data are normalised relative to pre-exercise fluorescence (B) Panel shows a representative overlaid FL1 histogram of CD4⁺ cells Pre (grey fill) and Post+0 (white fill) treated with 0.1 μ M F5M. CD4⁺T-Reduced⁺ was identified based on a highly reduced population of cells on the far right of the histogram (C) Bars represent changes in the concentration of CD4⁺T-Reduced⁺ cells per μ L in RA (grey bars) and TR (white bars) participants in response to acute exercise. All values are means \pm standard error.

Fig 5: Flow cytometric analysis of intracellular thiol redox status in CD8⁺ cells in response to acute exercise (n=20). (A) Bars represent normalised MFI on FL1-H (λ 488nm; λ 525/30nm) in gated CD8⁺ cells for RA (grey bars) and TR (white bars) participants. Data are normalised relative to pre-exercise fluorescence (B) Panel shows a representative overlaid FL1 histogram of CD8⁺ cells Pre (grey fill) and Post+0 (white fill) treated with 0.1 μ M F5M. CD8⁺T-Reduced⁺ was identified based on a highly reduced population of cells on the far right of the histogram (C) Bars represent changes in the concentration of CD8⁺T-Reduced⁺ cells per μ L in RA (grey bars) and TR (white bars) participants in response to acute exercise. All values are means \pm standard error. * indicates a significant difference relative to Pre: ** p<0.001. ⁺ indicates a significant difference relative to During: ++ p<0.001 (all Time*Group effect; pairwise comparisons).

Fig 6: Immunophenotypic analysis of $CD8^+T$ -Reduced⁺ activation, suppression and lymphoid homing following exhaustive exercise (n=6). (A) Chart illustrates changes in MFI for F5M in response to exercise (B-D) Bars represent MFI for PD-1 (B), CCR7 (C) and CD28 in $CD8^+T$ -Reduced⁺ (white bars) and $CD8^+T$ -Reduced⁻ (black bars) before and after exercise. Values are means \pm standard deviation. * indicates a significant difference relative to Pre (Time*Group effect; pairwise comparison): * p<0.05. # indicates a significant difference between $CD8^+T$ -Reduced⁺ and $CD8^+T$ -Reduced⁻ (Time*Group effect; pairwise comparison): # p<0.05.

References

1. **Badía M., Giraldo E, Dasí F, Alonso D, Lainez JM, Lloret A, Viña J.** Reductive stress in young healthy individuals at risk of Alzheimer disease. *Free Radic Biol Med* 63: 274–279, 13AD.
2. **Bergman BC, Brooks GA, Bryan C, Respira- GAB.** Respiratory gas-exchange ratios during graded exercise in fed and fasted trained and untrained men. .
3. **Brinkmann C, Blossfeld J, Pesch M, Krone B, Wiesiolek K, Capin D, Montiel G, Hellmich M, Bloch W, Brixius K.** Lipid-peroxidation and peroxiredoxin-overoxidation in the erythrocytes of non-insulin-dependent type 2 diabetic men during acute exercise. *Eur J Appl Physiol* 112: 2277–87, 2012.
4. **Campbell JP, Riddell NE, Burns VE, Turner M, van Zanten JJCSV, Drayson MT, Bosch J a.** Acute exercise mobilises CD8+ T lymphocytes exhibiting an effector-memory phenotype. *Brain Behav Immun* 23: 767–75, 2009.
5. **Cobley JN, Close GL, Bailey DM, Davison GW.** Exercise redox biochemistry: Conceptual, methodological and technical recommendations. *Redox Biol* 12: 540–548, 2017.
6. **Deminice R, Sicchiere T, Payao PO, Jordao A.** Blood and salivary oxidative stress biomarker following an acute session of resistance exercise in humans. *Int J Sport Med* 31: 599–603, 2010.
7. **Epps DE, Taylor BM.** A Competitive Fluorescence Assay to Measure the Reactivity of Compounds. *Anal Biochem* 295: 101–106, 2001.
8. **Ferrer MD, Tauler P, Sureda A, Tur JA, Pons A.** Antioxidant regulatory mechanisms in neutrophils and lymphocytes after intense exercise. *J Sports Sci* 27: 49–58, 2009.
9. **Forster R, Davalos-Misslitz AC, Rot A.** CCR7 and its ligands: balancing immunity

- and tolerance. *Nat Rev Immunol* 8: 362–371, 2008.
10. **Fry RW, Morton AR, Crawford GPM, Keast D.** Cell numbers and in vitro responses of leucocytes and lymphocyte subpopulations following maximal exercise and interval training sessions of different intensities. *Eur J Appl Physiol Occup Physiol* 64: 218–227, 1992.
 11. **Gomez-Cabrera MC, Domenech E, Romagnoli M, Arduini A, Borrás C, Pallardo F V., Sastre J, Viña J.** Oral administration of vitamin C decreases muscle mitochondrial biogenesis and hampers training-induced adaptations in endurance performance. [Online]. *Am J Clin Nutr* 87: 142–9, 2008. <http://www.ncbi.nlm.nih.gov/pubmed/18175748>.
 12. **Gomez-Cabrera MC, Domenech E, Vina J.** Moderate exercise is an antioxidant: upregulation of antioxidant genes by training. *Free Radic Biol Med* 44: 126–131, 2008.
 13. **Hadzic T, Li L, Cheng N, Walsh SA, Spitz DR, Knudson CM.** The Role of Low Molecular Weight Thiols in T Lymphocyte Proliferation and IL-2 Secretion. *J Immunol* 175: 7965–7972, 2005.
 14. **Hole PS, Zabkiewicz J, Munje C, Newton Z, Pearn L, White P, Marquez N, Hills RK, Burnett AK, Tonks A, Darley RL.** Overproduction of NOX-derived ROS in AML promotes proliferation and is associated with defective oxidative stress signaling. *Blood* 122: 3322–30, 2013.
 15. **Holmgren A, Lu J.** Thioredoxin and thioredoxin reductase: current research with special reference to human disease. *Biochem Biophys Res Commun* 396: 120–4, 2010.
 16. **Howley ET, Bassett Jr DR, Welch HG.** Criteria for maximal oxygen uptake: a review and commentary. *Med Sci Sport Exerc* 27: 1292–1301, 1995.
 17. **Ilie M, Falk AT, Butori C, Chamorey E, Bonnetaud C, Long E, Lassalle S, Zahaf K, Vénissac N, Mouroux J, Cohen C, Brambilla E, Marquette CH, Hofman V, Hofman P.** PD-L1 expression in basaloid squamous cell lung carcinoma: Relationship to PD-1+ and CD8+ tumor-infiltrating T cells and outcome. *Mod Pathol* 29: 1552–1564, 2016.
 18. **Ji LL.** Modulation of skeletal muscle antioxidant defense by exercise: Role of redox signaling. *Free Radic Biol Med* 44: 142–52, 2008.
 19. **Lamb GD, Westerblad H.** Acute effects of reactive oxygen and nitrogen species on the contractile function of skeletal muscle. *J Physiol* 589: 2119–2127, 2011.
 20. **Lloret, A., Fuchsberger, T., Giraldo, E., Vina J.** Reductive Stress: A New Concept in Alzheimer's Disease. *Curr Alzheimer Res* 13: 206–211, 2016.
 21. **Lu X, Yang L, Yao D, Wu X, Li J, Liu X, Deng L, Huang C, Wang Y, Li D LJ.** Tumor antigen-specific CD8+ T cells are negatively regulated by PD-1 and Tim-3 in human gastric cancer. *Cell Immunol.* .
 22. **Margaritelis N V., Kyparos A, Paschalis V, Theodorou AA, Panayiotou G, Zafeiridis A, Dipla K, Nikolaidis MG, Vrabas IS.** Reductive stress after exercise: The issue of redox individuality. *Redox Biol* 2: 520–528, 2014.
 23. **McArdle F, Pattwell DM, Vasilaki A, McArdle A, Jackson MJ.** Intracellular generation of reactive oxygen species by contracting skeletal muscle cells. *Free Radic Biol Med* 39: 651–7, 2005.
 24. **Michailidis Y, Karagounis, Leonidas G, Terzis G, Jamurtas, Athanasios Z,**

- Spengos K, Tsoukas D, Chatzinikolaou A, Mandalidis D, Stefanetti RJ, Papassotiriou I, Athanasopoulos S, Hawley JA, Russell, Aaron P, Fatouros, Ioannis G.** Thiol-based antioxidant supplementation alters human skeletal muscle signaling and attenuates its inflammatory response ... signaling and attenuates its inflammatory response and recovery after. *Am J Clin Nutr* 98: 233–45, 2013.
25. **Narasimhan M, Rajasekaran NS.** Reductive Potential - A Savior Turns Stressor in Protein Aggregation Cardiomyopathy. *Biochim Biophys Acta* 1852: 53–60, 2015.
 26. **Nielsen HB, Madsen P, Svendsen LB, Roach R., Secher NH.** The influence of PaO₂, pH and SaO₂ on maximal oxygen uptake. *Acta Physiol Scand* 164: 88–97, 1998.
 27. **Novák M, Procházka V, Turcsányi P, Papajík T.** Numbers of CD8+PD-1+ and CD4+PD-1+ Cells in Peripheral Blood of Patients with Chronic Lymphocytic Leukemia Are Independent of Binet Stage and Are Significantly Higher Compared to Healthy Volunteers. *Acta Haematol* 134: 208–14, 2015.
 28. **Petersen AC, Mckenna MJ, Medved I, Murphy KT, Brown MJ, Gatta P Della.** Infusion with the antioxidant N -acetylcysteine attenuates early adaptive responses to exercise in human skeletal muscle. *Acta Ohysiol* 204: 382–392, 2012.
 29. **Powers SK, Nelson WB, Hudson MB.** Exercise-induced oxidative stress in humans: Cause and consequences. *Free Radic Biol Med* 51: 942–950, 2011.
 30. **Ristow M, Birringer M, Kiehnopf M, Zarse K, Oberbach A, Klo N, Stumvoll M, Kahn CR, Blu M.** Antioxidants prevent health-promoting effects. *PNAS* 106: 8865–8670, 2009.
 31. **Sakelliou A, Fatouros IG, Athanailidis I, Tsoukas D, Chatzinikolaou A, Draganidis D, Jamurtas AZ, Liacos C, Papassotiriou I, Mandalidis D, Stamatelopoulos K, Dimopoulos MA, Mitrakou A.** Evidence of a Redox-Dependent Regulation of Immune Responses to Exercise-Induced Inflammation. *Oxidative Med.* .
 32. **Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A.** Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401: 708–712, 1999.
 33. **Salmeen A, Andersen JN, Myers MP, Meng T-C, Hinks JA, Tonks NK, Barford D.** Redox regulation of protein tyrosine phosphatase 1B involves a sulphenyl-amide intermediate. *Nature* 423: 769–773, 2003.
 34. **Samjoo IA, Safdar A, Hamadeh MJ, Raha S, Tarnopolsky MA.** The effect of endurance exercise on both skeletal muscle and systemic oxidative stress in previously sedentary obese men. *Nutr Diabetes* 3: e88, 2013.
 35. **Sang J, Yang K, Sun Y, Han Y, Cang H, Chen Y, Shi G, Wang K, Zhou J, Wang X, Yi J.** SUMO2 and SUMO3 transcription is differentially regulated by oxidative stress in an Sp1-dependent manner. *Biochem J* 498: 489–498, 2011.
 36. **Schafer FQ, Buettner GR.** Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. [Online]. *Free Radic Biol Med* 30: 1191–212, 2001. <http://www.ncbi.nlm.nih.gov/pubmed/11368918> [27 Jul. 2014].
 37. **Shing CM, Peake JM, Ahern SM, Strobel NA, Wilson G, Jenkins DG, Coombes JS.** The effect of consecutive days of exercise on markers of oxidative stress. *Appl Physiol Nutr Metab* 32, 2007.
 38. **Sjoback R, Nygren J, Kubusta M.** Absorption and fluorescence properties of

- fluorescein. *Spectrochim Acta* 51: L7–L21, 1995.
39. **Sobotta MC, Liou W, Stöcker S, Talwar D, Oehler M, Ruppert T, Scharf AND, Dick TP.** Peroxiredoxin-2 and STAT3 form a redox relay for H₂O₂ signaling. *Nat Chem Biol* 11: 64–70, 2014.
 40. **Souza Jr. TP, Oliveira PR, Pereira B.** Physical exercise and oxidative stress. Effect of intense physical exercise on the urinary chemiluminescence and plasmatic malondialdehyde. *Rev Bras Med Esporte* 11, 2005.
 41. **Steinbacher P, Eckl P.** Impact of Oxidative Stress on Exercising Skeletal Muscle. *Biomolecules* 5: 356–377, 2015.
 42. **Sureda A, Ferrer MD, Tauler P, Romaguera D, Drobnic F, Pujol P, Tur JA, Pons A.** Effects of exercise intensity on lymphocyte H₂O₂ production and antioxidant defences in soccer players. *Med, Br J Sport* 43: 186–190, 2009.
 43. **Tauler P, Sureda A, Cases N, Aguiló A, Rodríguez-Marroyo J a, Villa G, Tur J a, Pons A.** Increased lymphocyte antioxidant defences in response to exhaustive exercise do not prevent oxidative damage. *J Nutr Biochem* 17: 665–71, 2006.
 44. **Turner JE, Bennett SJ, Campbell JP, Bosch JA, Aldred S, Griffiths HR.** The antioxidant enzyme peroxiredoxin-2 is depleted in lymphocytes seven days after ultra-endurance exercise. [Online]. *Free Radic Res* 47: 821–8, 2013. <http://www.ncbi.nlm.nih.gov/pubmed/23889121> [19 Sep. 2013].
 45. **Turner JE, Bosch JA, Drayson MT, Aldred S.** Assessment of oxidative stress in lymphocytes with exercise. [Online]. *J Appl Physiol* 111: 206–11, 2011. <http://www.ncbi.nlm.nih.gov/pubmed/21493722> [4 Feb. 2013].
 46. **Veskoukisa AS, Nikolaidisb MG, Kyparosa A, Kouretas D.** Blood reflects tissue oxidative stress depending on biomarker and tissue studied. *Free Radic Biol Med* 47: 1371–1374, 2009.
 47. **Wadley AJ, Aldred S, Coles SJ.** An unexplored role for Peroxiredoxin in exercise-induced redox signalling ? *Redox Biol* 8: 51–58, 2016.
 48. **Wadley AJ, Chen YW, Bennett SJ, Lip GYH, Turner JE, Fisher JP, Aldred S.** Monitoring changes in thioredoxin and over-oxidised peroxiredoxin in response to exercise in humans. *Free Radic Res* 49: 290–298, 2015.
 49. **Wadley AJ, Chen YW, Lip GY., Fisher JP, Aldred S.** Low volume-high intensity interval exercise elicits anti-oxidant and anti-inflammatory effects in humans. *J Sports Sci* 27: 1–9, 2015.
 50. **Wherry EJ, Masopust D.** Adaptive Immunity. *Viral Pathog (3rd Ed* 5: 57–69, 2016.
 51. **Winterbourn CC.** Are free radicals involved in thiol-based redox signaling? *Free Radic Biol Med* 80: 164–170, 2015.
 52. **Winterbourn CC, Hampton MB.** Signaling via a peroxiredoxin sensor. *Nat Publ Gr* 11: 5–6, 2014.
 53. **Zuo Y, Xiang B, Yang J, Sun X, Wang Y, Cang H, Yi J.** Oxidative modification of caspase-9 facilitates its activation via disulfide-mediated interaction with Apaf-1. *Cell Res* : 449–457, 2009.